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***J. Clin. Microbiol.* 1986, 23(3):609.**

JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1986, p. 609–615
0095-1137/86/030609-07$02.00/0

Vol. 23, No. 3

Identification and Serotyping of *Microsporum canis* Isolates by Monoclonal Antibodies

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Received 26 August 1985/Accepted 6 December 1985

Hybridoma cells were produced by fusing mouse myeloma cells with spleen cells from mice primed with an exoantigen of *Microsporum canis*. Three clones produced antibodies which were examined by the Western blot technique for their potential usefulness in the identification of *M. canis* isolates and differentiation of strains within the species. Based on reactions with immunological determinants, all of the *M. canis* isolates tested presented either species- or strain-specific domains. Monoclonal antibodies proved to be useful reagents for the identification of *M. canis* isolates and for the differentiation of strains within the species. A purified antigen depleted of common antigenic determinants was obtained in affinity chromatography by using monoclonal antibody.

The application of hybridoma technology has proven to be useful in the identification of a wide range of microorganisms. Monoclonal antibodies may be used comparatively in different immunological assays for phylogenetic studies and species and strain identification.

In a previous report, we described the potential for serologically analyzing dermatophytes by precipitating monoclonal antibodies produced against an exoantigen of *Microsporum canis* (5).

This report deals with the properties of dermatophyte antigenic determinants as determined by the reaction of monoclonal antibodies with polypeptides separated by electrophoresis in polyacrylamide gels and then transferred electrically to nitrocellulose.

Purification of crude *M. canis* exoantigen by affinity chromatography with monoclonal antibodies might also be achieved to obtain antigens specific to *M. canis*.

MATERIALS AND METHODS

Cultures. Dermatophyte isolates were obtained from clinical specimens in our institute (Università Cattolica del Sacro Cuore; UCSC) or were graciously furnished by the Division of Mycotic Diseases, Centers for Disease Control, Atlanta, Ga. (see Tables 1 to 3). All the cultures were maintained in our collection at room temperature in sterile distilled water.

Reference antigens. *M. canis* CDC B2094 was the standard strain used for the production of the reference exoantigen and for mouse immunization. It was extracted in 24 h from a 7-day-old Sabouraud dextrose agar slant culture by using a merthiolate solution (1:5,000) in distilled water. The solution was filtered and concentrated 50-fold by lyophilization. The protein and carbohydrate contents of the reference antigen were adjusted to final concentrations of 2,500 and 550 μg/ml, respectively. The same procedure was used to produce exoantigens (protein content, 2,500 μg/ml) from all the cultures used in this study.

Immunization protocol. For the fusion, BALB/c mice were immunized intraperitoneally with 0.1 ml of the reference antigen mixed with an equal amount of incomplete Freund adjuvant. The procedure was repeated once a week for 1 month. Three days before the fusion, the mice were reinoculated intraperitoneally with 0.1 ml of the reference antigen only. Before the fusion, blood samples were collected from each mouse and placed in microtiter plates sensitized with the *M. canis* reference antigen for enzyme-linked immunosorbent assay of antibody titer.

Production and screening of hybridomas. The procedures for fusion and selection of the hybridoma cells producing monoclonal antibodies against *M. canis* exoantigen were performed as described elsewhere (5).

Western blot analysis. Dermatophyte lyophilized exoantigens (5 ml) were solubilized with 0.5 ml of disruptor buffer consisting of 1 M Tris (pH 7), 60% (wt/vol) sucrose, 2% (wt/vol) sodium dodecyl sulfate, 5% (wt/vol) B-mercaptoethanol, and 0.02% bromphenol blue-saturated solution. A 50-μg sample of protein standards (150 μl), including RNase A (13,700 daltons), chymotripsinogen (25,000 daltons), and ovalbumin (43,000 daltons), was added per ml of the antigen solution, and it was boiled for 5 min. Antigen samples were electrophoresed through 10% polyacrylamide gels (vertical gel system; Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Electrophoresis was performed at 15 mA and continued for 18 h to allow the solvent front to reach the bottom of the gel. Immunoblotting of the gels was performed by a modification of the method of Towbin et al. (6). Briefly, the proteins were electrically transferred to nitrocellulose sheets (0.45-μm pore size; Schleicher & Schuell, Inc., Keene, N.H.) at 60 V for 3 h (Trans blot cell; Bio-Rad Laboratories, Richmond, Calif.). After 90 min, the transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol) was changed. The nitrocellulose sheets were cut into strips and incubated with 5% horse serum in phosphate-buffered saline (PBS; pH 7.6) for 30 min at room temperature. The strips were subsequently incubated in a shaker for 30 min at 37°C in a 1:10 dilution of hybridoma culture fluid. The strips were then washed with PBS, blocked once more with 5% horse serum in PBS, and incubated for 30 min in a shaker at 37°C with 3 ml of horseradish peroxidase-coupled

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TABLE 1. Western blot analysis of dermatophyte isolates with *M. canis* monoclonal antibody UCSC 3

Isolate	Molecular size (kDa)
Epidermophyton floccosum	
CDC B3807	21 30 45
UCSC 1	30 45 58 84
UCSC 8	22 30 44 52 84
Microsporum audouinii	
CDC B3800	
M. canis	
CDC B2094	21 28 74 95
UCSC 1	19 23 74 80 95
UCSC 2	74 95
UCSC 4	25 60 74 95
UCSC 6	74 95
UCSC 8	74 95
UCSC 10	30 74 95
UCSC 12	37 48 74 95
UCSC 14	19 25 45 74 95
UCSC 52	25 40 74 95
M. cookei CDC B3803	89
M. distortum CDC B2174	72
M. equinum CDC B2699	
M. ferrugineum CDC 83-056097	66
M. fulvum UCSC 0	32 45 68 80
M. gallinae CDC B3801	
UCSC 0	31 42 50 68 90 110
M. gypseum	
CDC B3816	17 80
UCSC 0	18 24 75 84
UCSC 1	24 40 75
UCSC 2	24 45 65 85
UCSC 4	24 47 60
M. nanum CDC B3815	49 68 98
M. persicolor CDC B1923	38
M. racemosum UCSC 0	78
M. ripariae UCSC 0	86
Trichophyton megninii	20 37 76
UCSC 0	
T. mentagrophytes var. *erinacei*	62
CDC B1865	
T. mentagrophytes var. *interdigitale*	
UCSC 3	15 22 92
UCSC 5	24 31 42 62 92 120
UCSC 6	15 22 92
UCSC 7	14 24 46
T. mentagrophytes var. *mentagrophytes*	
CDC B6209	34 44 54 58 78
T. mentagrophytes var. *quinckeanum*	17 25 45
CDC B2331	

T. rubrum																													
CDC 3806																37		45							76	90			
UCSC 5	10														34								66						
UCSC 9						16									34							64					95		120
UCSC 10									21															75					
UCSC 11							18			22				33							60					90			
T. soudanense CDC 83-048430			13														40									90			
T. tonsurans CDC B3810		12			15																60								
T. tonsurans var. *sulphureum*								19			23			33						58								102	
UCSC 0																													
T. violaceum																													
CDC B3610					15																								
UCSC 2				14																									
UCSC 4				14								25																	
T. violaceum var. *glabrum*													27						56							90			
UCSC 0																													

rabbit anti-mouse immunoglobulin G antiserum (1:500 in PBS containing 5% horse serum). After being washed for 30 min with two changes of PBS, the strips were stained with 100 ml of distilled water containing 1 ml of a 1% solution of 4-chloro-1-naphthol and 0.01% hydrogen peroxide. A few strips were differentially stained with Coomassie blue for evaluating protein marker migration in the gel.

Reverse-phase HPLC. All common reagents were of analytical grade. The acetronile was Chromasolv grade purchased from Riedel-De Haen (Seelze-Hannover, Hannover, Federal Republic of Germany), and trifluoroacetic acid was from Sigma Chemical Co. (St. Louis, Mo.). The high-performance liquid chromatography (HPLC) water was bidistillation filtered, before use, on a Gelman GA6 Metricel membrane filter (0.45-μm pore size). Each exoantigen (5 lyophilized ml) was dissolved in 0.10 ml of solution A for HPLC; 0.010 ml was then used for each HPLC analysis. The HPLC conditions were as follows. HPLC column, Aquapore RP300 (Brownlee Laboratories, Santa Clara, Calif.); 220 by 4.1 plus 30 by 4.1 mm guard column; solution A, 90% H_2O–10% CH_3CN plus trifluoroacetic acid (0.1% [wt/vol]); solution B, CH_3CN (100%) plus trifluoroacetic acid (0.1% [wt/vol]). Gradient applied, percentage B: 0%, 1 min; 0 to 70% in 18 min; 70%, 2 min; reequilibration, 0% at 8 min; wavelength, 220 nm; adsorbance scale, 1.280. The HPLC apparatus was a two-pump Perkin-Elmer LC10 equipped with an LC85 UV-Vis spectrophotometric recorder and LC autocontrol, connected with a LKB two-channel recorder.

Affinity chromatography. All common reagents were analytical grade: Affigel 10 was purchased from Bio-Rad, and ethanolamine was purchased from Carlo Erba (Milan, Italy). Ascitic fluid (5 ml) of *M. canis* monoclonal antibody UCSC 7 was partially purified by precipitation with ammonium sulfate (50% saturation) at 4°C overnight. The pellet was collected after centrifugation (2,200 × *g* for 15 min at 4°C) and dissolved in 5 ml of a potassium phosphate buffer (0.05 mol/liter [ph 7.44; coupling buffer]). After dialysis against the buffer (three changes, 200 ×, 4°C), the protein concentration was determined by the method of Lowry et al. (3). The coupling was achieved by adding 2.5 ml of Affigel 10 slurry to 5 ml of antibody solution (48 mg of protein). Before use, the Affigel 10 was filtered on Whatman no. 1 cellulose paper and gently washed with 20 ml of cold distilled water. The suspension was stirred at 4°C for 6 h. Then 200 μl of an ethanolamine HCl solution (1 mol/liter; pH 8.0) was added to the suspension to ensure complete blocking of active ester groups which might have remained on the resin. After 1 h of stirring, the suspension was poured into a small column to obtain a chromatographic bed of 1 cm (height) by 2 cm^2 (area). The resin was exhaustively washed with the coupling buffer, the eluent A_{278} was checked by means of an LKB 2238 Uvicord SII connected to an LKB 2210 two-channel recorder. The uncoupled antibody was detached from the resin by washing the bed first with a sodium phosphate buffer (0.05 mol/liter; pH 7.11; buffer A) and then with NaCl (4 mol/liter in buffer A [buffer B]). This buffer sequence was the same as that subsequently used for the affinity chromatography. The resin was then reequilibrated by buffer A. A 120-μl sample of the reference antigen (approximately 300 and 66 μg of protein and carbohydrate content, respectively) was applied to the resin bed and allowed to stand for 4 h at 4°C. Washing with buffer A was performed to elute the unbound antigens until the eluent A_{278} reached the baseline. Then the antigen against the monoclonal antibody was separated by elution of the resin with buffer B. The purified antigen was collected and dialyzed against buffer A (three

TABLE 2. Western blot analysis of dermatophyte isolates with *M. canis* monoclonal antibody UCSC 7

Isolate	Molecular size (kDa)
Epidermophyton floccosum	
CDC B3807	17
UCSC 1	37 50
UCSC 8	24 33 47
Microsporum audouinii	
CDC B3800	14
M. canis	
CDC B2094	98
UCSC 1	22 25 80 98
UCSC 2	98
UCSC 4	75 98
UCSC 6	64 75 98
UCSC 8	98
UCSC 10	27 32 98
UCSC 12	98
UCSC 14	98
UCSC 52	24 75 98
M. cookei CDC B3803	13 45 90
M. distortum CDC B2174	16
M. equinum CDC B2699	11 21
M. ferrugineum CDC 83-056097	11
M. fulvum UCSC 0	25
M. gallinae CDC B3801	22 68
UCSC 0	86
M. gypseum	
CDC B3816	11 25 40
UCSC 0	25 34
UCSC 1	25
UCSC 2	25 30
UCSC 4	25 38 78
M. nanum CDC B3815	35 45 80
M. persicolor CDC B1923	35 45 78
M. racemosum UCSC 0	20 32 50 86 96
M. ripariae UCSC 0	21
Trichophyton megninii	
UCSC 0	33 50
T. mentagrophytes var. *erinacei*	65
CDC B1865	
T. mentagrophytes var. *interdigitale*	
UCSC 3	15 40
UCSC 5	18 52
UCSC 6	14 40
UCSC 7	17 58
T. mentagrophytes var. *mentagrophytes*	
CDC B6209	13 40 43 50 55 78
T. mentagrophytes var. *quinckeanum*	
CDC B2331	17 37 84

T. rubrum					
CDC 3806	14	29	45		
UCSC 5	10				
UCSC 9	22	33	56	61	100
UCSC 10	21	62	74		
UCSC 11	15	29	45		
T. soudanense CDC 83-048430	15	44			
T. tonsurans CDC B3810	12				
T. tonsurans var. *sulphureum*	19	46			
UCSC 0					
T. violaceum					
CDC B3610	20				
UCSC 2	14	30	41	62	
UCSC 4	14	30	41	62	
T. violaceum var. *glabrum*					
UCSC 0					

changes, 200 ×, 4°C). The unbound, purified antigen was collected and lyophilized. The resin was used repeatedly without loss of binding activity.

Immunospot assay. A modification of the procedure of Hawkes et al. (2) for the assay of antibodies, based on the application of the antigen as a spot to nitrocellulose paper, was used to detect the reactivity of immunoadsorbed monoclonal antibody in affinity chromatography with either bound or unbound antigen.

RESULTS

Three hybridoma cell lines producing monoclonal antibodies of the immunoglobulin G class were selected according to their different reactivity with the reference antigen as detected by the Western blot technique. This procedure also permitted study of the properties of the dermatophyte proteins with the monoclonal antibodies. Our results were as follows.

M. canis monoclonal antibody UCSC 3 recognized all of the *M. canis* isolates tested by reaction with specific antigenic determinants of 74 and 95 kilodaltons (kDa) (Table 1). The detection of reactive antigenic determinants of different molecular weights in the different isolates within the species suggests the existence of numerous serotypes. No heterologous dermatophyte species, showing cross-reactivity, exhibited the simultaneous presence of the 74- and 95-kDa antigenic determinants.

M. canis monoclonal antibody UCSC 7 characterized single *M. canis* isolated by either single species-specific (98-kDa) or strain-specific antigenic determinants of different molecular weights (Table 2). It was interesting to note that the five *M. gypseum* isolates tested showed a common domain of 25 kDa.

M. canis monoclonal antibody UCSC 13 (Fig. 1) proved to be the most interesting and specific one. It reacted only with a single 16-kDa antigenic determinant in each *M. canis* isolate tested. This reaction was also shared by the *Microsporum distortum* isolate tested, which concurs with the identity of the two species as demonstrated previously by mating experiments (4). Cross-reactions, although at different molecular weights, were observed only in the *Microsporum ferrugineum*, *Microsporum gallinae*, and *Trichophyton soudanense* isolates tested. These species are considered strictly related morphologically (Table 3).

Affinity chromatography was successful in purifying one

TABLE 3. Western blot analysis of dermatophyte isolates[a] with *M. canis* monoclonal antibody UCSC 13

Isolate	Molecular size (kDa)			
M. canis				
CDC B2094			16	
UCSC 1			16	
UCSC 2			16	
UCSC 4			16	
UCSC 6			16	
UCSC 8			16	
UCSC 10			16	
UCSC 12			16	
UCSC 14			16	
UCSC 52			16	
M. distortum CDC B2174			16	
M. ferrugineum CDC 83-056097	11			
M. gallinae CDC B3801				23
T. soudanense CDC 83-048430		12		

[a] The other dermatophytes listed in Tables 1 and 2 were negative with this monoclonal antibody.

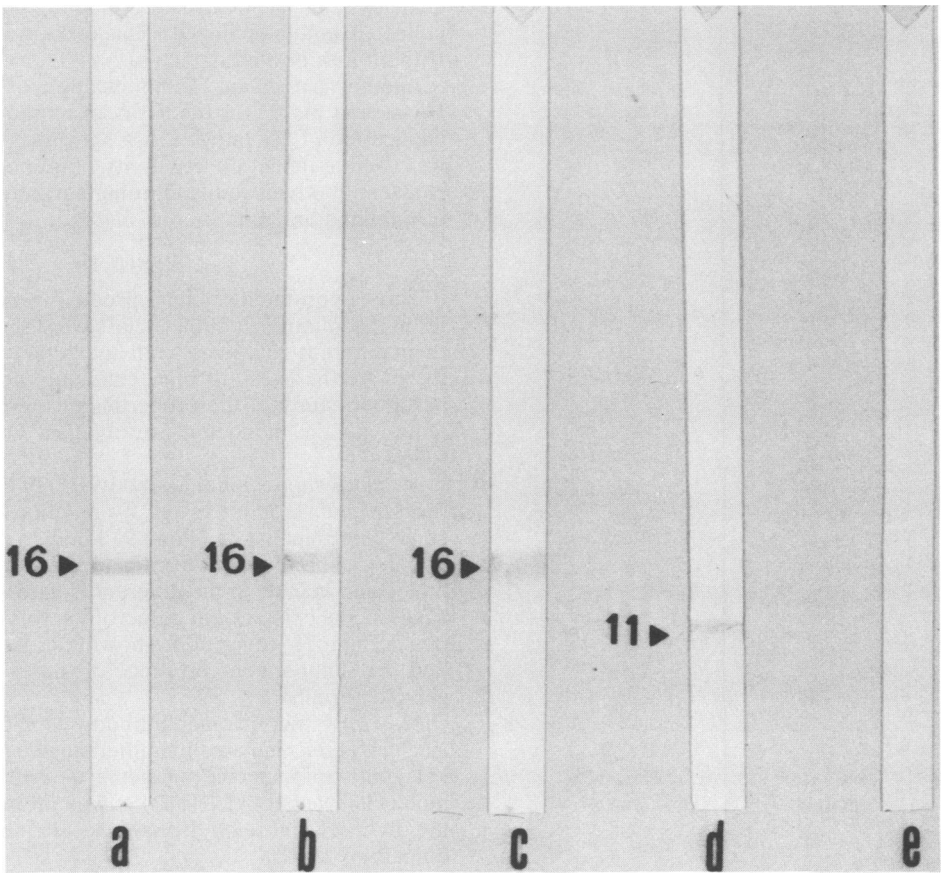

FIG. 1. Western blot analysis of dermatophyte isolates with *M. canis* monoclonal antibody UCSC 13 (molecular size expressed in kDa). Lanes: a, *M. canis* CDC B2094 exoantigen; b, *M. canis* UCSC 1 exoantigen; c, *M. canis* UCSC 12 exoantigen; d, *M. ferrugineum* CDC 83-056097 exoantigen; e, *Microsporum audouinii* CDC B3800 exoantigen.

component of the reference antigen. In a preliminary approach using *M. canis* monoclonal antibody UCSC 7 as an immunoadsorbent, it was possible to separate peak 1 (unadsorbed portion of the antigen) and peak 2 (adsorbed portion of the antigen) (Fig. 2).

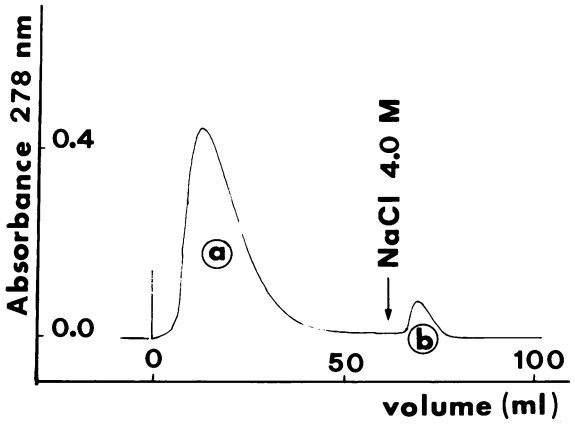

FIG. 2. Affinity chromatography of *M. canis* CDC B2094 total exoantigen against monoclonal antibody UCSC 7 coupled to Affigel 10 resin. Peak a represents the unadsorbed antigenic fraction, and peak b represents the fraction eluted by sodium chloride (4.0 M) in elution buffer (arrow).

The immunospot assay performed with the reference antigen in toto, peak 1 (unadsorbed antigen), and peak 2 (adsorbed antigen) with *M. canis* monoclonal antibody UCSC 7 showed the loss of reactivity of peak 1 (unadsorbed antigen) (Fig. 3).

Comparison by reverse-phase HPLC of the reference

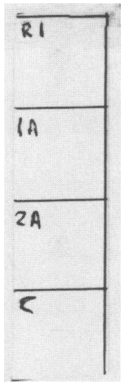

FIG. 3. Evaluation of reaction of in toto (R1), unadsorbed (1A), and adsorbed (2A) exoantigen of *M. canis* CDC B2094 by immunospot with *M. canis* monoclonal antibody UCSC 7. Antigenic fractions 1A and 2A were obtained after affinity chromatography of the exoantigen with *M. canis* monoclonal antibody UCSC 7. Control (C) was PBS used in place of the antigen.

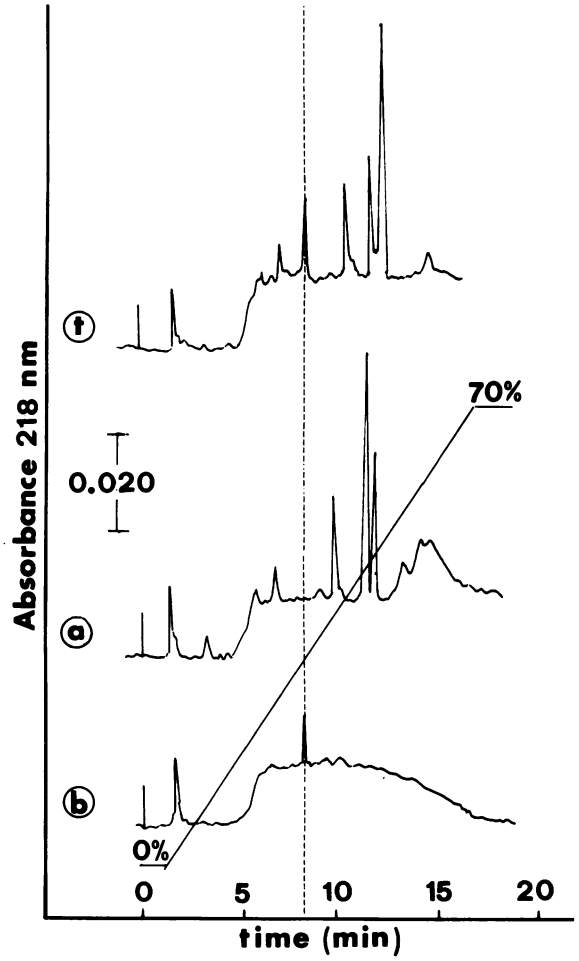

FIG. 4. Reverse-phase HPLC. t, *M. canis* CDC B2094 total exoantigen; a, unadsorbed antigenic fraction; b, the fraction eluted by sodium chloride (4.0 M) in elution buffer after affinity chromatography against monoclonal antibody UCSC 7 on Affigel 10 resin. The dashed line shows the position of the reactive antigenic determinant in the chromatographic pattern. The acetonitrile gradient applied in the chromatography is also indicated.

antigen and peak 1 (portion eluted) confirmed the disappearance of one peak of the original pattern (Fig. 4).

DISCUSSION

Our study clearly showed the enormous potential of monoclonal antibodies for the immunoidentification and typing of dermatophytes. The effectiveness of the monoclonal antibodies may be completely evaluated by using a highly sensitive and analytical procedure, such as the Western blot technique. It is of primary importance, moreover, that the immunization antigen be an exoantigen which can very easily and rapidly be produced from the dermatophyte isolates to be identified.

When the dermatophyte antigens were electrophoretically separated in denaturing gels and then immobilized on nitrocellulose strips, we detected a greater diversity of monoclonal antibody reactivity to fungal proteins than when we used the technique of immunodiffusion of soluble nondenaturated dermatophyte antigens (5). Analogous to the results obtained by Braun et al. (1) in the study of viral proteins, the preliminary advantage of the technique is in the detection of nonprecipitating antibody and of antibody to poorly soluble antigens.

The finding that multiple-molecular-weight components of the exoantigen reacted with monoclonal antibodies might suggest either modifications of a common protein or the polymerized states of a protein which was not separated under the denaturing conditions used in this study. Finally, the procedure of affinity chromatography by the immunoadsorption of nonspecific monoclonal antibodies might lead to the purification of antigens specific to *M. canis*.

ACKNOWLEDGMENTS

This study was supported by grants from the Ministero Pubblica Istruzione and Consiglio Nazionale delle Ricerche (Progetto Finalizzato: CMI Contratto no. 84.02040.52).

We thank Libero Ajello for his editorial assistance in the preparation of this manuscript.

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